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THE RESPONSE OF THE CHICKEN THYMUS TO THYROXIN

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled 'The response of the chicken thymus to thyroxin' submitted by Dale E. Alsager in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

The size of the thymus is influenced by the sex of the animal and the activity of the thyroid. The interdependence of these influences and their relationships to the nervous system have been tested.

The hen's thymus enlarges in response to exogenous thyroxin, but the cock's thymus is less responsive. The removal of both superior cervical sympathetic ganglia at hatching improves the response of the male. Removal of one ganglion is less effective. The effect of the unilateral operation is symmetrical; the thymus on the unoperated side is the same size as the thymus on the operated side. This indicates that the sympathetic system can restrain the response of the thymus to hormones, and it suggests that the restraint is exerted by means other than the direct sympathetic innervation of the thymus. This interpretation was also tested by cutting a possible alternate route of thymic innervation (the spinal nerves) which leads directly to the lobes of the thymus. This did not improve the response of the male's thymus to thyroxin, although it may have reduced the response of the female's thymus.

This work shows that the sympathetic system can restrain the response of the thymus to thyroxin, a hormone which stimulates the immunological response to antigens, and indicates that the restraint is not mediated by the direct innervation of the thymus.

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INTRODUCTION

It is increasingly clear that the thymus plays an important role in the maturation of some kinds of immunological competence (Miller and Osoba, 1967). There is little doubt that its presence assists natural resistance (Pari, 1906) and the production of antibodies (Scapaticci, 1938). Moreover, these immunological functions can be impaired by hormonal disturbances which affect the thymus (Manning, 1959; Santisteban, 1960; Bearn, 1960, 1961, 1966; Pepper, 1961; Scherzer, Azar, Naujoks, and Williams, 1963; Elders, Parham, and Hughes, 1968). These indications of the importance of the hormonal milieu are reinforced by differences between the sexes: the thymus of the female of some species is larger than that of the male (Santisteban, 1960; Ito and Hoshino, 1963; Scherzer *et al.*, 1963), and the female may be immunologically more competent than the male (von Haam and Rosenfeld, 1942; Ruth, Höhn, and Law, 1965; Washburn, Medearis, and Child, 1965). It is then not too surprising that estrogens can stimulate the thymus (Ruth, 1960) and androgens can inhibit it (Gregoire, 1945; Auerbach, 1966). Estrogens may not, however, be the most effective hormonal stimulants. The great seasonal enlargements of the avian thymus do not coincide with seasonal gametogenesis (Höhn, 1956), and the only treatment which simulates these enlargements is the administration of thyroxin (Höhn, 1959). This simulation is shown in this report to be sex dependent; it is inhibited in the male. The male is released from this inhibition by removal of the superior cervical sympathetic ganglia at hatching.

METHODS AND MATERIALS

All chickens were line #94, White Leghorn hybrids hatched from eggs supplied by Hy-Line Poultry Farms, Johnston, Iowa. All primary operations were done within two hours of hatching under a dissecting microscope equipped with a foot-operated focus and a fibre optics illuminator. Secondary exploratory operations were done when size was no longer a critical aspect of surgery. Two stimulants were used: adrenaline was injected prior to some primary operations and atropine was injected prior to exploratory operations. With experience the time required for primary operations was reduced and adrenaline proved unnecessary. Atropine, administered intramuscularly (0.0007 gr per kg), inhibited salivation and nasopharyngeal mucous, which are serious problems during exploratory operations.

All chickens were raised in group cages under an electrically regulated 14 hour day length.

Initially, chicks were anesthetized with Combutol in 0.85 per cent saline (Church, 1957). Since the difference between an effective and a lethal dosage proved small and unreliable, later operations were under Fluothane (see Appendix 6). Initially, adults were anesthetized by perfusion with Nembutol (60 mg per kg). This induced convulsions, spasms, and regurgitation and later operations were done under Combutol anesthesia; one fourth the total dosage was given in an initial, intravenous injection and the remainder was given in spaced, intraperitoneal injections.

The thymus is best exposed by mid-ventral incision. There are no direct principal connections between the ventral wall of the neck and the principal vessels, nerves, and organs of the neck so that spreading or widening of a mid-ventral incision does not disrupt any physiological

communications with the thymus. The thymus consists of six or seven bilateral pairs of separate lobes which form two parasagittal rows, one on each side of the neck. They are immediately obvious following the spreading of a mid-ventral incision: the most anterior pair of lobes lies close to the head and the most posterior pair lies close to the thyroid and the heart (Fig. 1). The lobes appear attached to the dorsolateral walls by light fascia and each lobe has its own medial connections to the vascular system and its own connection to the spinal cord by a spinal nerve. With their connections, each pair of lobes forms one transverse element in a repetitive series which extends the full length of the neck. The spinal nerves which pass through each lobe (Fig. 2), the superior cervical sympathetic ganglia which lie anterior to the thymus, and cranial nerves IX and X were approached through mid-ventral incisions. The exploratory operations also began with mid-ventral incisions. The only operation which did not begin with a mid-ventral incision was the transection of the sympathetic chains which were approached through lateral incisions.

Prior to each operation, feathers were plucked under light anesthesia, the bird was secured on its back or side, and the site was washed with alcohol. For ganglionectomy the neck was extended as far as possible to permit location of the greater cornu of the hyoid by palpation before incision. For transection of the sympathetic trunk, a lateral mid-cervical incision exposed the vertebral muscles permitting the location of the lateral processes of the vertebrae by direct palpation.

For ganglionectomy, the mid-ventral incision extended from the midpoint of the neck to the mandible. The superficial fascia covering the hyoid were cut and the hyoid was displaced laterally, exposing the jugular vein and nerves IX and X (Figs. 3 and 4). These were cleaned

forward to their exits from the skull. The jugular vein overlies the carotid artery which can be seen beneath deep fascia and between the two <u>rectus capitus ventralis</u> muscles (Fig. 5). The fascia were cut and the internal carotid artery was traced forward toward nerves IX and X near which it enters the skull (Figs. 6 and 7). Just short of this entry the internal carotid passes close to the large, opaque superior cervical sympathetic ganglion which can now be isolated by blunt dissection with fine forceps (Figs. 7 and 8). The ganglion can be removed, but the experimental data are based on its destruction by electrocoagulation. For transection of the sympathetic trunk the vertebral muscles were separated to expose the lateral process of a vertebra, which was cut (Fig. 9). This revealed the vertebral artery and the sympathetic trunk, which could then be cut without damage to the artery (Fig. 10). The spinal nerves to the thymus lobes were accessible for electrocoagulation after the lobes were freed of their fascia (Fig. 2).

Mid-ventral incisions were made at different levels of the neck to permit transection of cranial nerves anterior to, midway along, and posterior to the thymus. This was done primarily to facilitate a histological study of changes in cellular morphology of the thymus as a direct result of specific operations (see Appendix 2). Nerve X was transected at three levels: near its exit from the skull, between the cranial lobes of the thymus and the communication with nerve IX, and below the clavicle where nerve X approaches the thyroid. Nerve IX was transected at two levels: near the superior cervical sympathetic ganglion and halfway down the neck where nerve IX leaves the jugular vein and passes to the esophagus.

All primary operations, i.e., all transections of nervous connections, were done on newly hatched chicks and the incisions were closed

with a modified Glover's stitch using atraumatic non-cutting needles and 5-0 chromic gut. These sutures may be left in place indefinitely. All exploratory operations were done on adults and the incisions were closed with vertical running mattress sutures of 3-0 surgical silk. Penicillin and streptomycin were administered to prevent infection. Operated chicks were kept on heating pads until they recovered after which they were put in an incubator for eight to 24 hours (39°C, 85 per cent relative humidity). Adults recover readily without special precautions.

Thyroxin was administered to young adults and adults by injection into the <u>vena thoracica externa</u> anywhere along its course on the lateral body wall (Fig. 13). Each bird received 15 injections of 0.5 mg sodium L-thyroxin (National Biochemical Corporation, Cleveland, Ohio) suspended in 0.5 ml of 0.85 per cent saline. (Injection technique described in Appendix 3.) The injections were given on alternate days so that the birds received a total of 7.5 mg during a 30 day period. The technique for repetitive intravenous injection into the <u>vena thoracica externa</u> avoided three common difficulties: (1) tissue inflammation evoked by extravenous injection of thyroxin dissolved in oil (Höhn, 1959), (2) alteration of the venous drainage evoked by repetitive, unavoidably damaging, injections of the fragile veins of the wings, and (3) technical difficulties encountered by conventional injection methods in attempting to inject a substance, which is insoluble in isotonic saline, into the blood stream.

RESULTS

1. External Effects of Operations

All operations were preceded by plucking of feathers, under light anesthesia, from the areas of incision. The regeneration of the feathers was normal except for those areas of the skin which overlaid cut spinal nerves. The feathers in the affected areas were sparse and this condition persisted for at least one year. The feathers which developed in these areas were wire-like in appearance, brittle and barbless (Fig. 12) (see Appendix 1). Since this effect was produced only at the levels of the neck corresponding to the cut nerves, and only on operated sides, it served as confirmation of the success of the operation.

The removal of a superior cervical sympathetic ganglion has an immediate effect on the upper eyelid of the operated side. The eyelid droops for at least a year (Fig. 16). This operation also causes an immediate fluffing of the head feathers on the operated side (Fig. 14). The immediacy and persistence of these effects are indicative of the success and permanence of the operation.

2. Effects of Thyroxin on Unoperated Chickens

A preliminary experiment with commercial chickens of both sexes showed that thyroxin stimulates the growth of the hen's thymus. (These birds were obtained from a local supplier and were of unknown background.) The male's thymus did not enlarge significantly. This demonstration of sex-dependence was not fully acceptable because the ages and histories of the birds were unknown and the repeated intramuscular injections of the oil used to dissolve thyroxin caused extensive necrosis. The experiment was redone with 42 closely related, 3-month old, line #94

hybrid White Leghorns hatched in the laboratory and raised under observation. Ten were untreated controls, 12 were injected intravenously with saline, and 20 were injected intravenously with thyroxin suspended in saline. The intravenous injections caused no obvious tissue damage or necrosis. Each saline control received 15 injections of 0.85 per cent saline and each experimental bird received 15 injections of 0.5 mg sodium thyroxin suspended in saline. The injections were given on alternate days and all birds were killed two days after the last injection.

Males weigh more than females and the data for the two sexes are separated (Table 1). The mean weights for controls represent untreated controls plus saline injected controls. Thyroxin did not alter the body weights significantly, but it increased the thymus weights of females 71 per cent ($p < 0.001$). When adjusted for the difference between control and treated body weights, the stimulation of the female's thymus is 80 per cent.

The thymus of the thyroxin treated female is only slightly larger than the thymuses of control and treated males. The explanation can be found in the last column of Table 1: The male's thymus is nearly as large as the female's because the male is a heavier bird. The treated female's thymus is 74 per cent heavier than the control male's and 48 per cent heavier than the treated male's, after adjustment for body weights ($P < 0.001$). The differences between control and treated males are non-significant.

Two treated males have very large thymuses which might suggest that these thymuses had responded to thyroxin. In order to assess the significance of these it is necessary to know more about the relationship of thymus weight to body weight. As indicated by the slopes of the

regressions for treated males and females (Table 2, 'b'), the thymus weight (Y) increases about 8 mg for each gm increase in body weight (X). This means that the presence of very large thymuses in a few treated males is not due to a specific stimulation of the thymus by thyroxin.

3. Section of the Spinal Nerves

Each thymus lobe is in contact with a spinal nerve which continues through it to the body wall. The intimacy of this contact suggested that these nerves might bear sympathetic fibres, from sympathetic rami (gray rami communicantes) to the thymus. These nerves were cut at hatching as a test of their importance to the thymus. Three months later exploratory operations were performed on 13 operated and 12 sham operated birds. Five operated birds had normal or large thymuses and eight had small thymuses, as judged by visual inspection. Eleven shams had normal thymuses and one had a small thymus. This suggests that cutting the spinal nerves inhibits the growth of the thymus of some birds (χ^2; $0.010 < p < 0.025$). This is at best only a suggestion since the numbers are small, visual inspection and weights can give very different impressions and reclassification of a single observation would make the Chi-square non-significant. Notwithstanding these reservations, the thymuses of operated birds seemed more variable than normal and the main source of this variation seemed to be the failure of some thymuses to grow normally. This led to direct tests for variation, alteration of normal growth, and the alteration of the response to thyroxin.

The effect of thyroxin on the thymus of operated birds was tested in two experiments (Table 3: 11 and 111). In experiment 11, 19 unoperated and 10 bilaterally operated, 3-month birds were separated into sub-groups: saline injected and thyroxin injected. At the end of treatment the ratio

of the mean thymus weight to the mean body weight for each 'saline' sub-group (X) was subtracted from the ratio for the corresponding 'thyroxin' sub-group (T) to give the 'thyroxin stimulation' (Y). Experiment III was a similar test of 11-month birds. In both experiments the 'thyroxin stimulation' of the females exceeded the 'thyroxin stimulation' of the males. This is best seen in the last column which represents the relative responses of the two sexes as the ratios of their 'thyroxin stimulations' (Y♀/ Y♂). The operation reduced this ratio from 4.11 to 1.38 for 3-month birds and from 3.48 to 1.76 for 11-month females. This reduction of the differences between the sexes suggests that the operation enhances the response of the male's thymus to thyroxin. Comparison of the 'thyroxin stimulations' for the two sexes shows that this is not so. The greatest male stimulation is 1.03 and the smallest female stimulation is 1.08, and the means for the two sexes are significantly different. The operation does not enhance the response of the male's thymus.

The interpretation of experiments II and III is complicated by the low ratios of thymus to body weight and by the low 'thyroxin stimulations' of the females. The fundamental difficulty is evident in the regressions at the bottom of Table 3. The 'thyroxin stimulation' (Y) of the male is not affected by variation of 'X', but the 'thyroxin stimulation' of the female is very dependent on 'X', the ratio of thymus to body weight for the saline controls. When this control ratio is small the 'thyroxin stimulation' is small and when this ratio is large the 'thyroxin stimulation' is large. It is not possible to distinguish the effect of the operation on 'thyroxin stimulation' from this effect of low thymus weight on 'thyroxin stimulation'. For this reason the reduced response of the operated female should be regarded as uncertain.

The exploratory inspections and the later tests indicate that the cutting of the spinal nerves causes variation in the normal growth and 'thyroxin stimulation' of the thymus. If this effect is a specific consequence of interrupting the innervation of the thymus, the lobes on the cut side should be more variable than those on the uncut side. The left spinal nerves of eight males and six females were cut at hatching. Three months later four males and three females were injected with saline and equal numbers were injected with thyroxin. Two days after the last injection the right and left thymuses were weighed separately (Table 4). The left and right thymuses are very similar. The regression at the bottom of Table 4 represents the regression of the left thymus weight (Y) on the right thymus weight (X). The coefficient of correlation is 0.98; $p < 0.001$. This very high correlation indicates that a comparison of the left thymuses with the right thymuses should detect effects specific for the left thymus. This is attempted in Table 4 by representing the left thymus as a percentile of the right thymus. The mean percentile and standard error for males is 109 ± 1.9 and that for females is 109 ± 11.8. The identity of the means and the dissimilarity of their standard errors show that the variation between the left and right parts of the female's thymus is greater than that of the male's ($F = 29.0$; $P < 0.001$). This suggests that the operation may have effects on the thymus of the female.

4. Superior Cervical Sympathetic Ganglionectomy

Experiments II to IV indicate that the lesser response of the male's thymus to thyroxin is not due to a repression communicated by direct innervation from the cervical spinal nerves and the rami which seem to connect these nerves with the sympathetic chains. This suggests that the effects, if any, of the sympathetic system on the response of

the male's thymus must be mediated by more indirect means. To test this a preliminary experiment was done with fourteen 5-month birds. Three males and three females were kept as unoperated, untreated controls. Five males and three females were used as experimental birds. The experimental birds differed from the controls in two ways: both superior cervical sympathetic ganglia had been removed at hatching and these birds received 15 injections of 0.5 mg sodium thyroxin beginning at 5 months. Two days after the last injection the mean for the thymuses (mg/gm) of experimental males exceeded that for control males by a factor of 2.93 and that for experimental females exceeded that for control females by a factor of 2.90. The mean for experimental males was 108 per cent of the mean for experimental females. The removal of both superior cervical sympathetic ganglia at hatching made the males fully as responsive as the females. If the experiment's mean is used to distinguish negative from positive, all control weights are negative and all experimental weights are positive.

On the basis of this preliminary experiment, 94 birds were used for a more formal test of the influence of the superior cervical sympathetic ganglia (experiment V). In this experiment, as in I to IV, there is a special consideration to which attention has not previously been called. The amount of thyroxin injected is the same for all birds irrespective of body weight. This means that the females, which are smaller, received more thyroxin per kg than the males. Were it not for the preliminary experiment in which the amount of thyroxin per kg was constant, the use of higher concentrations in the female would not be justified. The difference in concentration, in mg per kg, should have an effect since the 3-month male outweighs the female by one-third and

therefore receives a stimulus three-fourths as intense. This difference serves, however, as a precaution against overdosing the male in experiments designed primarily to test the existence of a normal repression of the male's response to thyroxin. Thus, if the operated male responds well to a concentration of thyroxin which is only three-fourths of that which causes a good response by the female, there seems little doubt that the operation has lifted a repression of the male's response.

Fifty-four males and 40 females were divided into six subgroups: unoperated and untreated controls, unilaterally operated and untreated, bilaterally operated and untreated, unoperated and treated, unilaterally operated and treated, and bilaterally operated and treated (experiment V: Table 5). Operated birds were those from which one or both superior cervical sympathetic ganglia had been removed at hatching. Treated birds were those injected with 7.5 mg sodium thyroxin given in 15 equal injections. All thymuses were weighed two days after the last injection. The data are presented as $\log_{10}$ mg/gm because this transformation makes the mean variances for the operated, treated males comparable to that for the unoperated, untreated males. The means for the unilateral, treated males and the bilateral, treated males are not significantly greater than that for the unoperated, untreated males ($0.100 < p < 0.200$), but they are significantly greater than the mean for 'all other' males (all unoperated or untreated males: lines 2, 4, 6, and 8, Table 5; $p < 0.001$). The increase in the response of the male due to the unilateral removal of one superior cervical sympathetic ganglion at hatching is about two-thirds of the increase obtained after removal of both ganglia. The removal of both ganglia makes the male 85 per cent as responsive as the unoperated female and 70 per cent as responsive as the bilaterally oper-

ated female, relative to their respective controls. This corresponds well with the mean difference in the concentration of sodium thyroxin administered to males and females; the concentration administered to males was 72 per cent of that administered to females.

DISCUSSION

It has been known for some time that thyroxin in moderate amounts can stimulate the growth and differentiation of the thymus (Gyllensten, 1953). This has been confirmed for the female chicken (Höhn, 1959). Unlike the classical demonstrations of many endocrine effects there is no report of the degree to which this effect is dependent on the nervous system. It seemed desirable, therefore, to test the possibility that the nervous system may influence the thyroxin-responsiveness of the thymus, if only to dismiss the possibility. The experiment became more attractive when a preliminary experiment showed that the female chicken is more responsive than the male. This observation provided an unusual opportunity because it made it feasible to undertake an experiment which could answer the question in either of two contrasting ways: alteration of the nervous system might depress the greater sensitivity of the female or it might enhance the lesser sensitivity of the male. If the first were correct the result would be open to the criticism that manipulation of the nervous system might have excited a general stress, a serious qualification of any experiment designed to demonstrate a specific effect on the thymus. On the other hand, an enhancement of the response of the male to thyroxin would not be open to this criticism since enhancement of the thyroxin-response would be the opposite of the effect of stress.

Thyroxin and estrogens are the only endocrine hormones which are known to stimulate the thymus (Gyllensten, 1953; Höhn, 1959; Ruth, 1960) and it is possible that they act in synergy when present in physiological or moderate concentrations. More importantly, the androgens are known depressants of the thymus (Gregoire, 1945; Auerbach, 1966) and it

is possible that the androgens of the male chicken inhibit thyroxin-stimulation of the thymus. Thus the difference in the responsiveness of the two sexes might be due solely to humoral or endocrine differences. A manipulation of the nervous system which reduces the difference in the thyroxin responsiveness of the sexes may act by altering the levels of circulating androgen or estrogen. The levels of androgen and estrogen in the birds used here were not determined, but the external sex characteristics and the behaviour of these birds were not noticeably altered by any of the operations. It is inferred, consequently, that the results of the experiments are not due to major alterations of the humoral mechanisms which govern sexual development. This inference receives some support from the timing of the operations. These were done at hatching, when sexual development had hardly begun. The thyroxin-responsiveness was tested at three months or later when the external characteristics of the sexes are definitive. Had there been any major shifts in the elaboration of sex hormones this should have been expressed by the occurrence of birds of intermediate sexual character. Such birds were not seen. The sexes were easily separable in all experimental and control groups and it is therefore unlikely that any of the results represent intersexes or transformed birds. For this reason it is felt that the data represent the different thyroxin-sensitivities of the thymuses of the two sexes without fundamental alteration of their other differences.

Since the experiments were undertaken with two explicit, alternate answers in mind—a reduction of the thyroxin-responsiveness of the female or an enhancement of the thyroxin-responsiveness of the male—it was desirable to bias the experiments against both answers. This was

done by injecting males and females alike with a fixed dosage of thyroxin so that the heavier males received a lower concentration of thyroxin in mg per kg body weight, about 75 per cent of that administered to the females. Thus, if an operation on the nervous system depressed the female's response to thyroxin without depressing the size of the male's thymus there would be little doubt that the effect is properly represented as a specific inhibition of the 'thyroxin stimulation' of the female. Conversely, if an operation enhances the male's response to thyroxin without enhancing the female's response there is little doubt that the effect is properly represented as a specific release of 'thyroxin stimulation' from an inhibition normally imposed by the male.

Experiment I (Tables 1 to 3) was a simple test of the response of unoperated, 3-month birds. The relationship of thymus weight to body weight is given in Table 2 which shows that the slopes of the regressions of thymus weight on body weight are the same for thyroxin-treated males and females. The close similarity of the slopes suggests that the thymuses of males and females respond to thyroxin in similar ways although the females are about 43 per cent more responsive than males of the same weight.

Experiments II and III show that the interruption of the spinal nerves which pass through or very close to each lobe of the thymus may interfere with the thyroxin-response of the female, but it is difficult to interpret the data to mean that operation has a specific effect. The regressions at the bottom of Table 3 indicate that the thyroxin-response of the female thymus may be abnormally low because the thymuses were abnormally small prior to treatment, as indicated by the thymus weights of untreated females. Thus it is not possible to infer that the operation specifically interferes with the thyroxin-responsiveness of the female's

thymus.

Experiment IV tested the effect of cutting the left spinal nerves. Irrespective of treatment the thymus on the operated side was similar to the thymus on the unoperated side. The left thymus is nine per cent larger than the thymus on the right and this difference has been confirmed in other experiments with operated and unoperated birds. This is the normal difference and the operation does not account for it. A peculiarity of this experiment is the indication that the left and right thymuses of females are less similar than those of the male, but the similarity is still very great. The similarity of the two sides suggests that the thymus is under a general humoral control which affects both sides to the same degree, with the qualification that the left thymus is slightly larger than the right. Experiment IV is the best indication that the dissection and weighing of the thymus is not a major source of random error in these experiments. If it were it is almost inconceivable that the unilateral operation could produce the nearly perfect correlation of the right and left thymus weights reported in Table 4.

A number of attempts were made, other than interruption of the spinal nerves, to alter the thymus. One of these, the removal of both superior cervical sympathetic ganglia, gave promising results. These ganglia were removed from five males and three females at hatching and the birds were treated with thyroxin at five months. The mean thymus weight for males, in mg per gm, was slightly greater than that for females and both means were three times the means for the unoperated, untreated controls. The concluding experiment is based on this preliminary observation.

The final experiment showed that the removal of both ganglia enhances the male's response to thyroxin, and that removal of one has a

lesser effect. The response of the bilaterally operated male is comparable to that of the unoperated female. The experiment also shows that the operated female is at least as responsive as the unoperated female. The experiment meets the two questions formulated earlier. The sex difference can be reduced by operative manipulation of the nervous system, and the manipulation stimulates the male's response instead of depressing the female's—the more acceptable alternative.

Experiment V suggests that the sympathetic system may mediate the lesser responsiveness of the male's thymus to thyroxin and may therefore act to inhibit the thymus. It is difficult to translate effects on organ size to effects on organ function, particularly in the case of the thymus, an organ the functions of which are not understood. In general, the size of the thymus reflects the state of the lymphoid and immune systems and a large, non-tumorous, thymus is indicative of good health and immunological competence. Since the differentiation of thymus lymphocytes and circulating lymphocytes is stimulated by thyroxin (Ernström, 1965) and the thymus is of great importance in the resistance of the animal to environmental infection (Bealmer, 1967; Miller, et al., 1967), any significant alterations of the response of the thymus to thyroxin have potential significance for immunology.

There is increasing evidence that the female is immunologically more competent than the male and it is well known that the female suffers a higher incidence of auto-immune disorders (Ruth, Höhn, and Law, 1965). It may then be of general importance that the thymus of the female chicken is sensitive to thyroxin and of more importance that the removal of the superior cervical sympathetic ganglia makes the male nearly as sensitive. It is not obvious, however, how this restraint is exerted nor is it clear

that it is the ganglia as such rather than the sympathetic system which exert this restraint. It may be that any alteration of the sympathetic chains would produce the same results or, conversely, it may be that the restraint is exercised specifically by the fibres from the superior cervical sympathetic ganglia to the pituitary or other endocrine glands. The present experiments show that a manipulation of the sympathetic system can alter the response of the thymus to hormone, but they do not exclude the possibility that manipulations of other parts of the sympathetic or even the parasympathetic system might not have similar effects. A positive, stimulating effect on the thymus has been demonstrated, but the functional importance of the demonstration is unknown.

SUMMARY

1. Hybrid chickens hatched in the laboratory and raised under controlled conditions were treated with an aqueous suspension of sodium thyroxin given as 15 small injections into the vena thoracica externa. This procedure avoids the mechanical problems, necrosis, and vascular disruption which follow the more usual modes of administration.

2. The female's thymus enlarged in response to thyroxin. The male's thymus was less responsive.

3. Females and males were submitted at hatching to operative alterations of the peripheral nervous system in an attempt to reduce the difference between the sexes either by reducing the responsiveness of the female or by enhancing the responsiveness of the male. The experiments were biased against a reduction of the female's response and against an enhancement of the male's response by using the same total dosage of thyroxin for all birds, the smaller females and the larger males.

4. None of the operations reduced the responsiveness of the female's thymus in a way which could be interpreted as specific.

5. One of the operations, the removal of one or both superior cervical sympathetic ganglia, increased the response of the male's thymus from zero to 85 per cent of the response of the unoperated female and 70 per cent of the response of the operated female. The difference between operated males and females is comparable to the difference in

the amount of thyroxin administered per kg.

6. The removal of the superior cervical sympathetic ganglia reduces the sex difference in thyroxin responsiveness by releasing the male thymus from an undefined inhibition. It is not known whether this inhibition depends specifically on the presence of the ganglia or on the intactness of the sympathetic chains.

7. This is the first report of the greater response of the normal female's thymus to thyroxin and the first report of the stimulation of the thymus response by operative manipulation of the autonomic nervous system. The effect of the operation does not seem to be related to interference with the autonomic control of the thymus vasculature, to post-operative stress, or to alteration of the gross differences between the sexes.

Table 1

Experiment 1

Stimulation of the Female Thymus by Thyroxin$^{\delta}$

Groups	n	Body Wt. (gm)	Thymus Wt. (mg)	Thymus Wt./Body Wt.
All ♂	24	1438 ± 25	6503 ± 401	
All ♀	18	1107 ± 27***	5911 ± 542^{ns}	
Control ♂	12	1464 ± 43	6120 ± 489	4.13 ± 0.30
Thyroxin ♂	12	1410 ± 37^{ns}	6885 ± 630^{ns}	4.85 ± 0.40^{ns}
Control ♀	10	1143 ± 32	4489 ± 511	3.98 ± 0.48
Thyroxin ♀	8	1063 ± 41^{ns}	7690 ± 610***	7.19 ± 0.49***

ns: non-significant; $0.050 < p$.

***: very highly significant, $p < 0.001$.

δ : Untreated and saline injected controls are pooled because they are very similar.

Note: all ns and *** refer to comparisons with preceding line.

Table 2

Experiment I

Regression of Thymus Wt. on Body Wt. $y = \bar{y} + b(x - \bar{x})$;

y = Thymus Wt. in mg; X = Body Wt. in gms.

Groups	n	b	F	r	p
Control ♂	12	7.44	6.92*	0.64*	$0.010 < p < 0.025$
Control ♀	10	-3.12	0.32^{ns}	0.20^{ns}	$0.500 < p$
Thyroxin ♂	12	8.10	2.90^{ns}	0.47^{ns}	$0.100 < p < 0.200$
Thyroxin ♀	8	8.20	2.67^{ns}	0.49^{ns}	$0.100 < p$ 0.200
Thyroxin ♂ + Thyroxin ♀ after adjustment of means	20	8.36	7.88*	0.55*	$0.010 < p < 0.025$

Table 3

Experiments I, II, and III

Effect of Bilateral transection of Spinal Nerves in the Response of the Thymus to Thyroxin

Expt.	Age (months)	Sex	n	Operation	Treatment* No Thyroxin X	Thyroxin	Stimulation Y	$\frac{Y♀}{Y♂}$
I	3	♂	24	No	4.18*	4.88*	0.70	4.74
	3	♀	18	No	3.98	7.29	3.31	
II	3	♂	10	No	3.96	4.60	0.64	4.11
	3	♀	9	No	2.00	4.63	2.63	
	3	♂	4	Yes	4.26	5.29	1.03	1.38
	3	♀	6	Yes	1.38	2.80	1.42	
III	11	♂	10	No	1.11	1.42	0.31	3.48
	11	♀	6	No	0.56	1.64	1.08	
	11	♂	6	Yes	0.43	1.40	0.97	1.76
	11	♀	5	Yes	0.78	2.49	1.71	

Y♂ $= 0.73 + 0.03\ (X - 2.78)$; regression is non-significant.

Y♀ $= 2.03 + 0.62\ (X - 1.74)$; regression is significant. $0.010 < p < 0.025$] and 3 d f

*thymus wt.(mg) / body wt.(gm)

(mean mg per gram for the group)

Table 4

Experiment IV

Correlate Size of Left and Right Thymuses after Transection of Left Spinal Nerves

Sex	Treatment	Right Thymus (mg) X	Left Thymus (mg) Y	Per cent Difference 100 Y/X
♂	Saline	2130	2400	112.7
♂	"	1970	1970	100.0
♂	"	2740	2850	104.0
♂	"	1970	2250	114.2
♂	Thyroxin	5410	5880	108.7
♂	"	3800	4370	115.0
♂	"	4480	4980	111.2
♂	"	2720	2900	106.6
♀	Saline	1210	1510	125.8
♀	"	3180	2870	90.3
♀	"	1000	1400	140.0
♀	Thyroxin	4680	6000	128.2
♀	"	1080	1180	109.3
♀	"	1280	790	62.7

$Y_{♂} = 3450 + 1.12\ (X - 3153)$; $Y_{♀} = 2292 + 1.23\ (X - 2072)$; $r_{♂} = 1.00$; $r_{♀} = 0.96$.
$\bar{X} = 2689 \pm 385$; $\bar{Y} = 2953 \pm 457$; $Y = 2953 + 1.16\ (X - 2689)$; $F_{1,12} = 259$.
$r\ (♂ + ♀) = 0.98$; $p < 0.001$. Variability of operated thymus of ♀ is significantly greater than that of ♂; $F = 29.0_{5,7}$***

Table 5

Effect of Removing the Superior Cervical Sympathetic Ganglia:

Stimulation of the Thymus of Operated Males by Thyroxin

Live	Operation	Thyroxin	Sex	n	$\log_{10}$ $\frac{\text{Thymus Wt(mg)}}{\text{Body Wt(gm)}}$ $\bar{y} \pm$ S.E.	$\frac{\bar{y}\text{ Experimental}}{\bar{y}\text{ Control}}$ ♀	♂
1	No	No	♀	10	0.66060 ± 0.04417	1.000	-
2	No	No	♂	8	0.55616 ± 0.04729	-	1.000
3	Unilateral	No	♀	5	0.61771 ± 0.08392	0.935	-
4	Unilateral	No	♂	10	0.52793 ± 0.04820	-	0.949
5	Bilateral	No	♀	6	0.58201 ± 0.06326	0.881	-
6	Bilateral	No	♂	9	0.52882 ± 0.05655	-	0.951
7	No	Yes	♀	8	0.82062 ± 0.03983	1.242*	-
8	No	Yes	♂	8	0.53756 ± 0.06051	-	0.967
9	Unilateral	Yes	♀	5	0.88211 ± 0.03080	1.335*	-
10	Unilateral	Yes	♂	10	0.65431 ± 0.03423	-	1.176***
11	Bilateral	Yes	♀	6	0.87000 ± 0.04556	1.317*	
12	Bilateral	Yes	♂	9	0.70731 ± 0.04238	-	1.272***

* : significantly greater than control.

*** : very highly significant when compared with all males of lines 2, 4, 6, and 8.

Figure 1. Location of the thymus in the neonatal chicken. The left thymus (lower pink pointer) lies along the left jugular vein (blue pointer). The right thymus has a similar location but is not shown. The skin has been removed from the left dorsal aspect of the neck so that the left thymus could be photographed against the blue background. Yellow pointer: esophagus. Upper pink pointer: trachea.

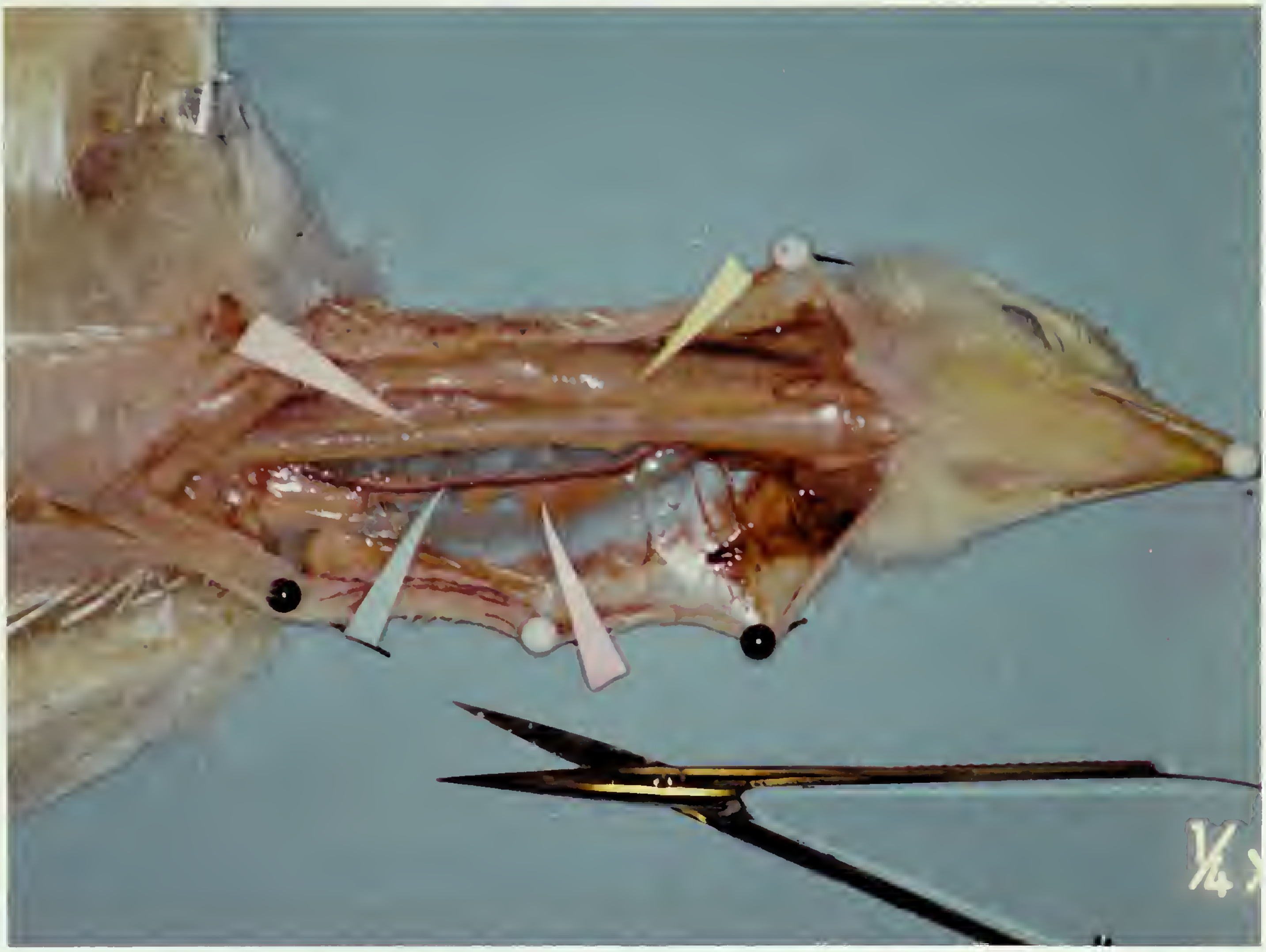

1/4 x

Figure 2. Relationship of the cervical spinal nerves to the thymus in the one week old chick. The left thymus lobes lie between the jugular vein and the dorsal skin. The perivertebral musculature is seen near the bottom of the figure just above the white pinhead. The spinal nerves pass through these muscles to the jugular and the thymus lobes and then to the skin. Pointer: a spinal nerve. (x 3)

Figure 3. The operative field revealed by a mid-ventral incision in preparation for removal of the superior cervical sympathetic ganglion. The superficial sub-dorsal fascia are intact. The beak is beyond the left of the figure. Probe: indicates anterior edge of cornu of hyoid. Forceps: retracts esophagus and trachea. Pointer: left jugular vein flanked by cranial nerves IX and X. (x 4)

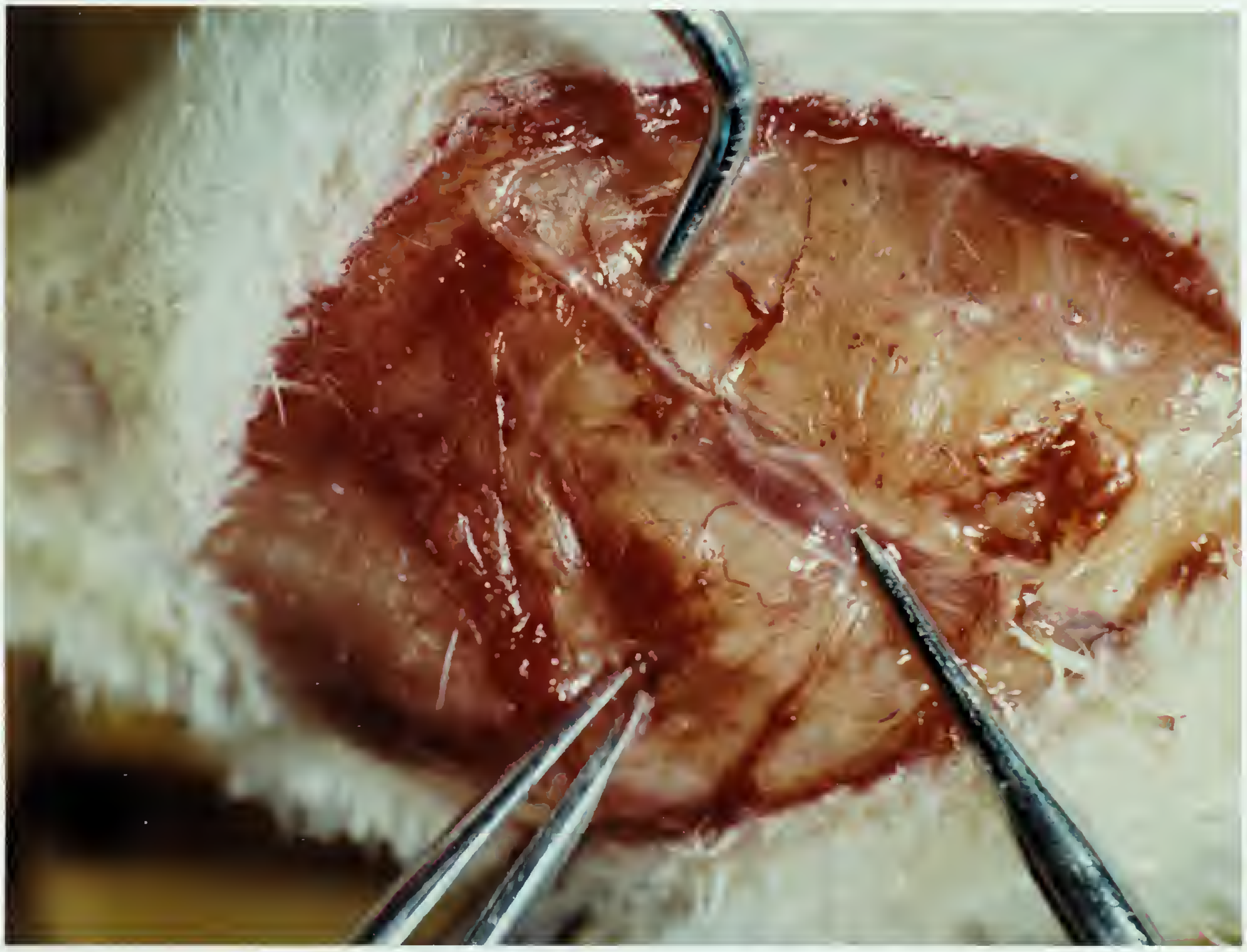


Figure 4. Operative field for removal of the superior cervical ganglion following incision of the superficial fascia overlying the jugular vein (probe).

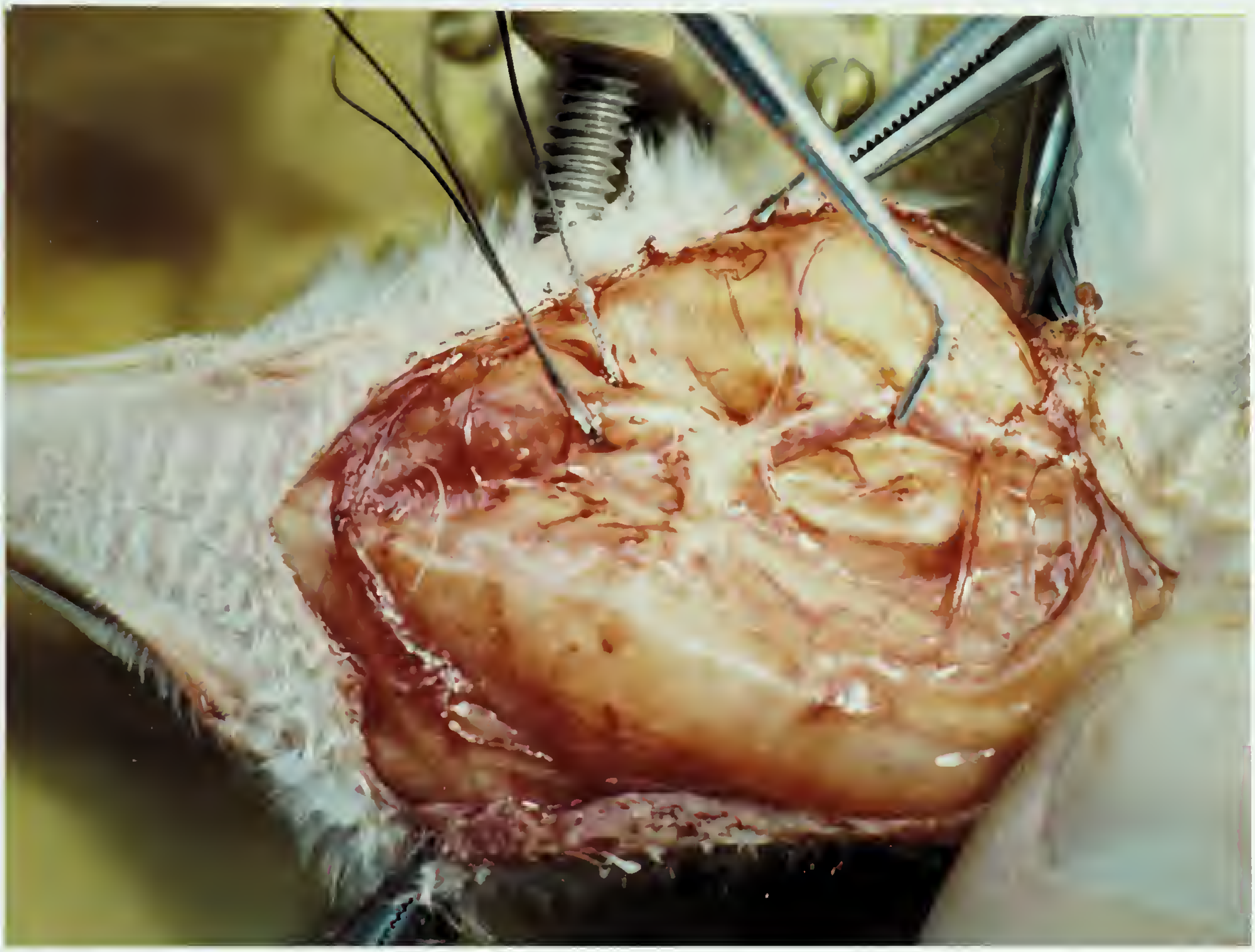
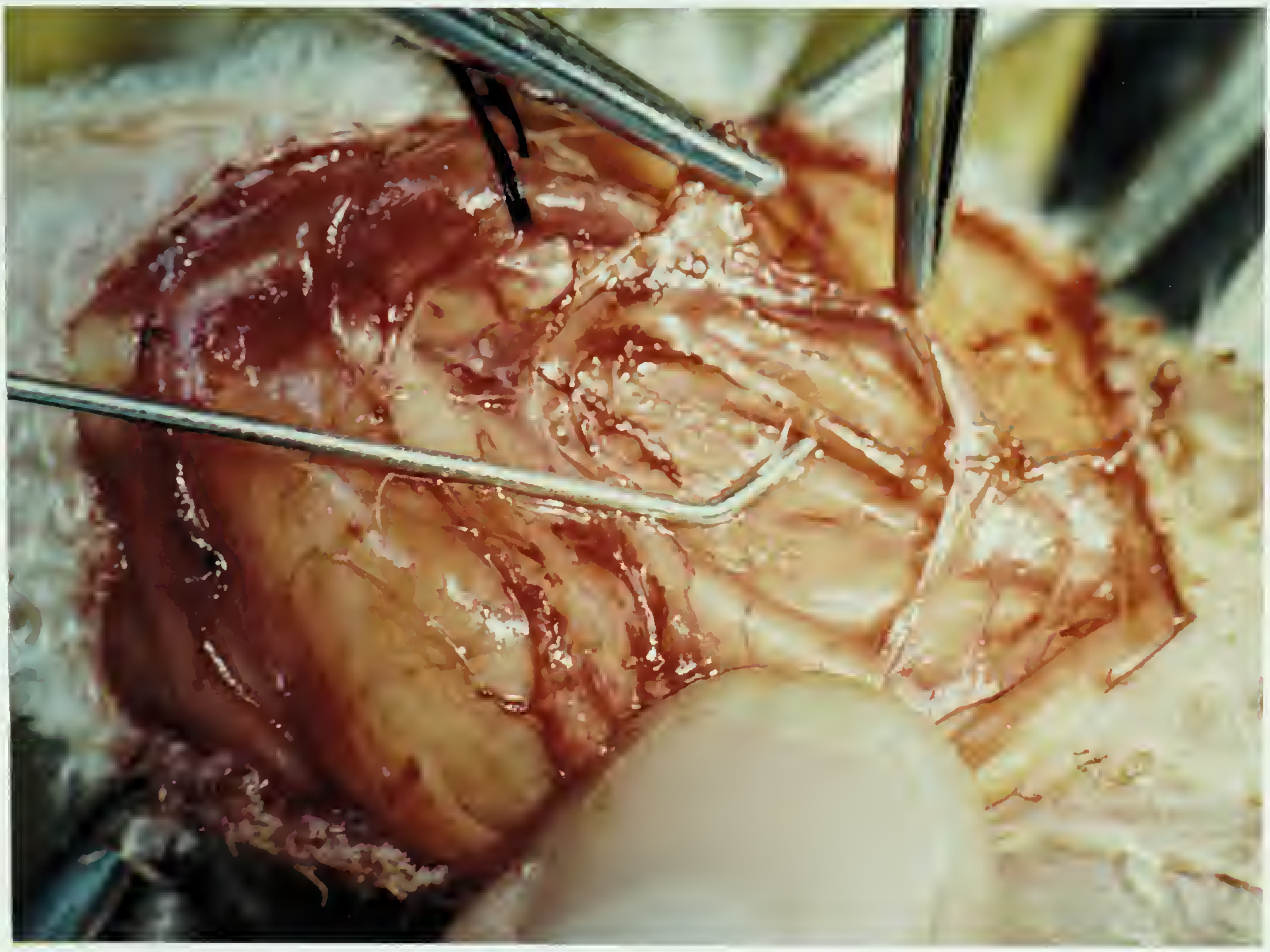


Figure 5. Location of the carotid artery preliminary to locating the superior cervical sympathetic ganglion. The cornu of the hyoid is retracted by a suture and the jugular is displaced up and to the left by the two pieces of forceps. The tip of the probe indicates the carotid artery 3X.



Figures 6 and 7. Tracing the internal carotid artery to the superior cervical sympathetic ganglion.

Figure 6. The cornu of the hyoid is displaced to reveal the v-shaped bifurcation of the left common carotid artery to form the internal and external carotid arteries. The external carotid lies just above the probe and the internal carotid is close to the tip of the forceps. Cranial nerve X lies at the tip of the forceps and parallels the internal carotid. Below and to the right of the probe the internal carotid gives off the sphenoid artery which curves under cranial nerve X.

Figure 7. The internal carotid and cranial nerve X have been cleaned to their exits from the cranium. The internal carotid lies just to the right of the probe's tip which touches the ganglion (pointer). The ganglion is wedged between cranial nerves IX and X. To the right of the ganglion (i.e. caudal) cranial nerve X gives off a branch which communicates with cranial nerve IX.

Figure 8. The left superior cervical sympathetic ganglion. The cornu of the hyoid curves across the upper part of the field. The internal carotid (pink pointer) passes beneath the nodasal ganglion, cranial nerve X (beneath brown-orange pointer), and the communication (tip of brown-orange pointer) between X and IX, and parallels cranial nerve IX (tip of blue pointer) to the lower limit of the field. The superior cervical sympathetic ganglion (green pointer) lies in the angle formed by nerves IX and X which have been separated to expose it. (x 18)

Figure 9. The operative field in preparation for transection of the sympathetic trunk. The pointers indicate the locations of two lateral processes of cervical vertebrae. Although the processes cannot be seen they are easily located by palpation of the perivertebral musculature. Both processes are cut to permit dissection of the field of Fig. 10.

Figure 10. The left sympathetic trunk revealed by dissection between the points indicated in Fig. 9. The sympathetic trunk crosses the pointer. The vertebral artery parallels the sympathetic trunk just beneath the pointer. The cut ends of the lateral processes lie on either side. (x 15)


A
B
1/3x

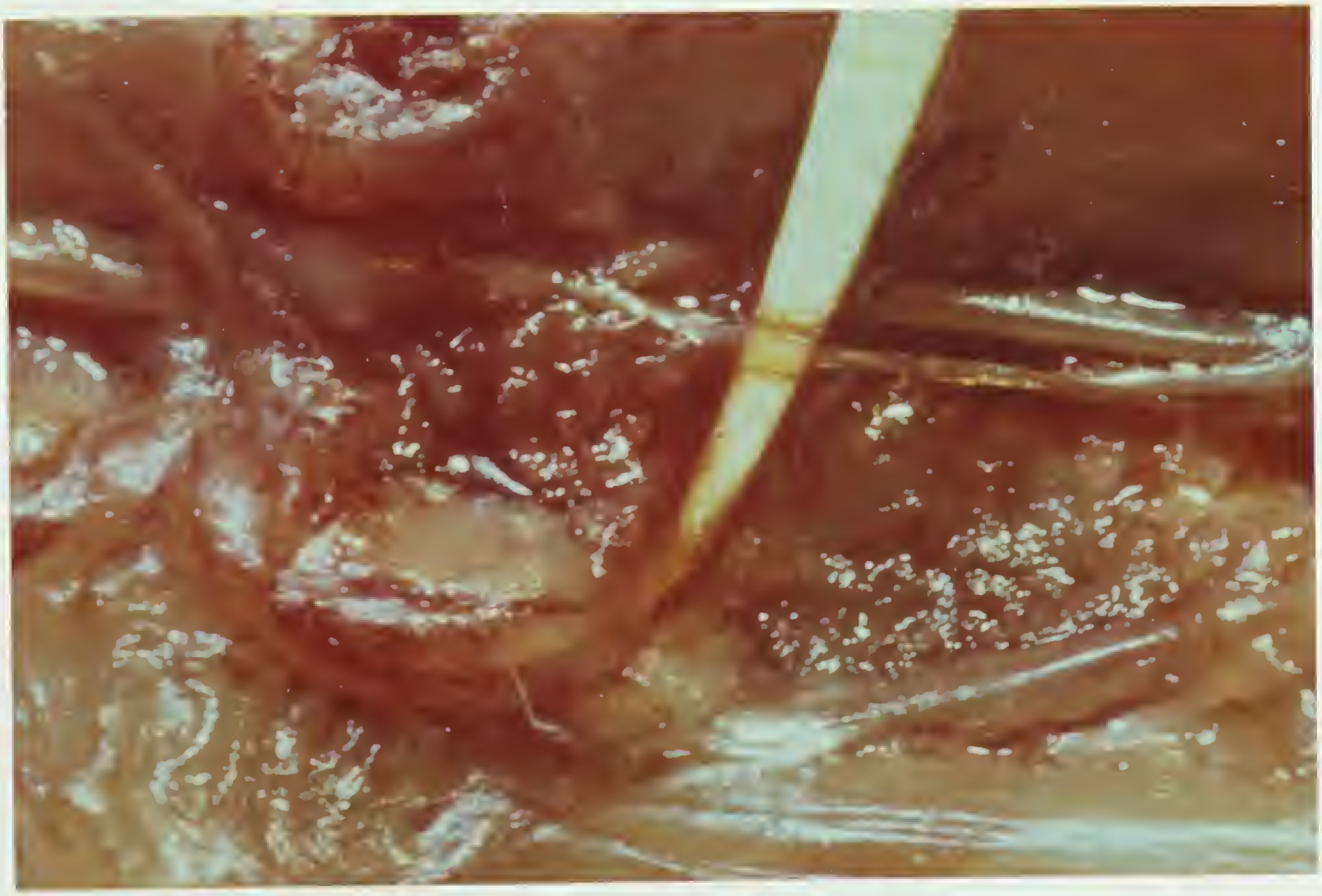


Figure 11. The ventral neck region of a 4-month old chicken. This bird received a sham operation for spinal nerve transection at hatching. Neck feathers have regenerated in a normal manner.

Figure 12. The ventral neck region of a 4-month old chicken whose cervical spinal nerves were transected at hatching. Feathers showed abnormal regeneration in all areas of skin innervated by the spinal nerves which were transected. At this age, only blackened stumps of feather shafts grew and rarely extended beyond the surface of the skin. In older birds the feathers which did grow were brittle and wire-like and lacked barbs.


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Figure 13. Injection of L-thyroxin Na into the right _vena thoracica externa_ of the chicken. Relatively low blood pressure in this vein facilitates repeated injections without haematoma.

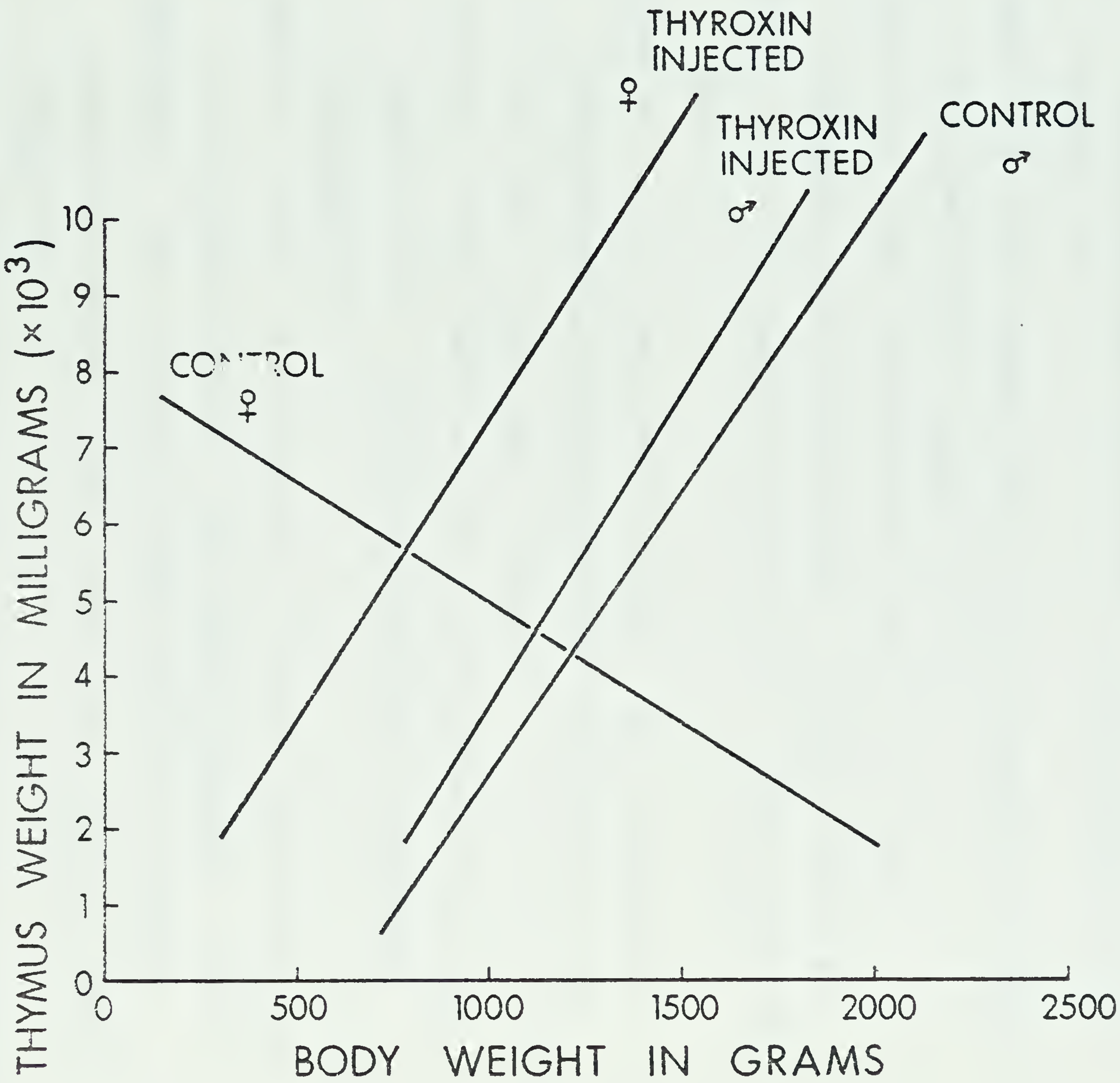
Figure 14. A newly hatched chick, unilaterally ganglionectomized (left) within two hours after hatching. Head feathers "stand up" notably and eyelid droops on the operated side.


1x

Figure 15. The right eye of a left unilateral sympathetic ganglionectomized chicken is normal at three months of age.

Figure 16. The left eye of a left unilateral sympathetic ganglionectomized chicken shows notable ptosis. This serves as evidence of the permanency and accuracy of the operation.

Figure 17 Regression plots of data from Table 2. This figure illustrates that thyroxin has significantly altered the growth of the female thymus after treatment with thyroxin. The male thymus, on the other hand, has not been significantly affected by thyroxin.


THYROXIN
INJECTED
♀
THYROXIN
INJECTED
♂
CONTROL
♂
CONTROL
♀
THYMUS WEIGHT IN MILLIGRAMS (× 10³)
0
1
2
3
4
5
6
7
8
9
10
0
500
1000
1500
2000
2500
BODY WEIGHT IN GRAMS

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APPENDIX 1

The Effect of Spinal Nerves on Feather Regeneration and Development

In summary:

1. At hatching the chicks were anesthetized and spinal nerves were sectioned: there were three groups of birds—bilateral denervation, unilateral denervations, and controls.
2. Unilaterally denervated birds showed some variation. About 3/4 of them showed retarded feather regrowth on the operated side. About 1/4 of them appeared to have normal feathers on both sides of the neck at 3 months. I suspect that this is due to an overlap or regeneration of nerves from the unoperated side.
3. At about 9 months to 1 year of age the feathers of bilaterally denervated birds appeared almost hair-like, with a very thin brittle shaft with very few or no barbs—except for a small tuft often present at the very end of the feathers.
4. Effect of Na Pentobarbitol on the feathers prior to plucking: Normally (on control birds) when birds are killed with an overdose of pentobarbitol the feathers are extremely easy to pluck out. However, denervated bird feather plucking was not facilitated with the use of pentobarbitol. In some cases the neck feathers were impossible to pull out by hand without tearing the skin. This was distinctly a local occurrence (ventral cervical area) since feathers elsewhere on the operated birds were easy to pluck.
5. As part of the above study of the thymus, thyroxin was given intravenously to half the bilateral, unilateral and control birds at 3 months of age and the injections (0.5 mg per bird) were repeated every second day for a month. The previously described effect on feather growth was unaltered in any way by the thyroxin

and appears to be completely independent of any thyroxin effects.

6. Irrespective of the thyroxin treatment, the feathers (of 3 month birds) on the ventral area of the neck appeared as if they had begun to grow and then started to degenerate from the inside. Blackish looking and abnormally shaped (open-ended) portions of feather shaft protruded from the skin. Often a very small new feather seems to be growing up beside the degenerate feather-shaft stub—almost appearing to come through the same opening in the skin, but growing around the existing shaft.

7. Histological sections of skin were prepared of denervated and control birds. Sections through skin and feather follicles seem to indicate: the dermal papillae were histologically normal excepting that the pulp did not extend above the level of the skin and the upper part was keratinized.

I have assumed from the literature and correspondence that this is the first time that this phenomenon has been demonstrated and supported by photographic evidence.

APPENDIX 2

The Effect of Surgical Operations on the Histology of the Thymus

HISTOLOGY OF THYMUS

At the ages specified in the text for the specific operative groups, thymus tissue was examined histologically for changes in gross and cellular morphology.

I. Tissue Preparation

Birds were sacrificed by rapid injection of a strong solution of out-dated Nembutol. Thymus samples were then quickly removed from upper cervical, central and lower cervical areas respectively and placed on ice in small, labelled aluminum dishes. Thymus tissues were then fixed depending on the method of sectioning to be used.

II. Fixation

a. Frozen Sections:

Fixation of tissues destined for frozen sectioning involved fixation in formal calcium fixative for 48 hours. Tissue samples were then transferred to gum sucrose for 24 hours and frozen in O.C.T. medium.

b. Wax Sections:

Some tissues (which were considered likely to be photographed later) were fixed for 48 hours in Carnoy fixative. After dehydration tissue sections were wax imbedded.

III. Sectioning

a. Frozen:

Sections were cut 3-6 microns thick from blocks of frozen O.C.T. imbedding medium containing the thymus tissues. Following sectioning on a Harris Cryostat at -20°C, tissue sections were mounted on labelled, gelatin-coated slides.

b. Wax:

Wax sections were cut 5-7 microns thick and placed on labelled, gelatin-coated glass slides.

IV. Staining

Tissues were then stained using a modification of the technique used by Stevens and Mainwaring (1967). This technique was modified in the following way:

1. Staining for 3-1/2 minutes in haematoxylin instead of only one minute.
2. Lithium solution was used as a blueing agent rather than ammonia.
3. Counter-staining in Eosin for 45 seconds.

V. Microscopy

Stained and mounted thymus sections were then studied microscopically for changes in gross and cellular morphology.

HISTOLOGICAL RESULTS

In order to establish a basis for histological comparisons of thymus tissue from various operated birds, a so-called 'normal' thymus histological morphology will be described first. The following description is observable in all controls of this experiment and no significant difference in morphology was noted between upper, central and lower cervical areas of the thymus.

A. Control 'Normal Thymus'

Cortex:

The peripheral, dark-staining cortex consists mainly of densely packed small lymphocytes with dark nuclei and, in between them, only a relatively few reticular cells, with pale staining nuclei.

Medulla:

In the medulla is the reverse. As one proceeds from the cortex toward the medulla, the number of lymphocytes drops rather abruptly, but there is no sharp line of demarcation between the two zones. The medulla is more vascular than the cortex. Here, where lymphocytes are less numerous, it can be seen that the reticular cells form a network with its meshes filled with lymphocytes.

Key:
- - - no effect on thymus
- + - slight effect
- ++ - average effect
- +++ - great effect
- * - abnormal feather regeneration on neck
- ⊙ - 'eyelid ptosis'
- † - loss of <u>pili</u> erector control in head feathers

HISTOLOGICAL RESULTS SUMMARY

Operative Type	Age at Observation	Gross Effect of Operation		Histological Effect of Operation	
		Unilateral	Bilateral	Unilateral	Bilateral
Unilateral Vagotomy above Thymus	4 weeks	-	-	-	-
	10-1/2 weeks	-	-	-	-
Unilateral Vagotomy high, near the Superior Cervical Sympathetic Ganglion	4 weeks	-	-	-	-
Sham Operated Control for above Vagotomy	4 weeks	-	-	-	-
Bilateral Vagotomy	(Lethal Operation)				

Histological Results Summary (Cont'd)

Operative Type	Age at Observation	Gross Effect of Operation		Histological Effect of Operation	
		Unilateral	Bilateral	Unilateral	Bilateral
Unilateral Vagotomy below Area 5 of the Thymus	12 weeks	-	-	-	-
Unilateral Glosso-pharyngeal Nervotomy above Thymus	4 weeks 11 weeks	- -	- -	- -	- -
Sham Operated Glossopharyngeal Nervotomy above Thymus	4 weeks	-	-	-	-
Bilateral Glosso-pharyngeal Nervotomy	4 weeks	-	-	-	-
Unilateral Glosso-pharyngeal Nervotomy as low as possible relative to Thymus	8 weeks	-	-	-	-
Unilateral Spinal Nervotomy	10-1/2 weeks	- *	-	-	-

Histological Results Summary (Cont'd)

Operative Type	Age at Observation	Gross Effect of Operation		Histological Effect of Operation	
		Unilateral	Bilateral	Unilateral	Bilateral
Control for Unilateral Spinal Nervotomy	10-1/2 weeks	-	-	-	-
Bilateral Spinal Nervotomy	4 weeks	- *	+++ *	-	++
Incomplete Bilateral Spinal Nervotomy	4 weeks	-	+++	-	+
Sham Operated Bilateral Spinal Nervotomy	4 weeks	-	-	-	-
Auto-transplanted Thymus (Unilateral)	10 weeks	+++	-	+++	-
Control for Unilateral Auto-transplant	10 weeks	-	-	-	-

<u>Histological Results Summary</u> (Cont'd)

Operative Type	Age at Observation	Gross Effect of Operation		Histological Effect of Operation	
		Unilateral	Bilateral	Unilateral	Bilateral
Bilateral Superior Cervical Sympathetic Ganglionectomy	3 weeks 6 weeks 8 weeks	- - -	⊙ † ⊙ † ⊙ †	- - -	- - -
Sham Operated Bilateral Superior Cervical Sympathetic Ganglionectomy	3 weeks 5-1/2 weeks 8 weeks	- - -	- - -	- - -	- - -
Unilateral Superior Cervical Sympathetic Ganglionectomy	6 months	++++ ⊙ †	-	+++	-
Sham Operated Unilateral Superior Cervical Sympathetic Ganglionectomy	6 months	† ⊙ -	-	-	-
Unilateral Sympathetic Trunk Lesion at mid-Cervical Level	9 weeks	† ⊙	-	-	-
Unilateral Sympathetic Trunk Lesion - sub-Thymic	8 weeks	† ⊙	-	-	-

Histological Results Summary (Cont'd)

Operative Type	Age at Observation	Gross Effect of Operation		Histological Effect of Operation	
		Unilateral	Bilateral	Unilateral	Bilateral
Sham Operated Unilateral Sympathetic Trunk Lesion	8 weeks	-	-	-	-
Bilateral Sympathetic Trunk Lesion	8 weeks	-	† ⊙	-	-
Sham Operated Bilateral Sympathetic Trunk Lesion	3 months	-	-	-	-
Combined Vagotomy and Glossopharyngeal Nervotomy (Unilateral)	4 weeks	-	-	-	-
Combined Vagotomy, Glossopharyngeal Nervotomy and Spinal Nervotomy (Unilateral)	4 weeks	-	-	-	-
Combined Vagotomy and Spinal Nervotomy (Unilateral)	4 weeks	-	-	-	-

Histological Results Summary (Cont'd)

Operative Type	Age at Observation	Gross Effect of Operation		Histological Effect of Operation	
		Unilateral	Bilateral	Unilateral	Bilateral
Unilateral Vagotomy and Sympathectomy	(Lethal Operation)				
Unilateral Vagotomy, Sympathectomy and Spinal Nervotomy	(Lethal Operation)				

Bilateral Sham for Sympathetic Trunk Lesions:

Three months after operation:

Gross Effects:

1. Thymus appeared normal in gross.
2. No other obvious effects.

Histology: Normal

COMBINATION DENERVATIONS

1. Unilateral Vagotomy and Glossopharyngeal Nervotomy:

   Four weeks after operation:

   Gross Effect: No change in Thymus, Bursa or Spleen in gross appearance.

   Histology: No change from normal cellular morphology on either side.

2. Unilateral Vagotomy, Glossopharyngeal Nervotomy and Spinal Nervotomy:

   Gross Effect: No change in Thymus, Bursa or Spleen in gross appearance.

   Histology: No effect due to operation.

3. Unilateral Vagotomy and Spinal Nervotomy:

   Gross Effect: Nil.

   Histology: Normal - comparable to control birds of this age.

4. Unilateral Vagotomy and Sympathectomy:

   - no birds survived.

5. Unilateral Vagotomy, Sympathectomy and Spinal Nervotomy:

   - no birds survived.

APPENDIX 3

Technique Used for Injection of Thyroxin or
Other Insoluble Substances Intravenously

AN IMPROVED TECHNIQUE FOR REPEATED INTRAVENOUS INJECTIONS IN FOWL

Introduction

A common problem in research laboratories utilizing fowl as an experimental animal, has been the inability of a vein to stand up under repeated intravenous injections. The following is a description of a method, whereby a modified technique of injection is used on a rather unusual vein, which invariably insures the integrity of the vessel throughout extended periods of repeated injections.

REVIEW OF CONVENTIONAL TECHNIQUES

In the past, this laboratory and probably many others, have used the large brachial vein located subcutaneously on the ventral of the wing for all intravenous injections. During an experiment where repeated injections of a suspension were required, 30 birds were injected in this manner every second day. After four or five injections, utilizing the veins on both wings, relatively small hypodermic needles (No. 26) and painstaking efforts to assist clotting, subcutaneous bleeding and blood blistering at the point of puncture of the vein actually threatened success of any further injections.

PROCEDURE OF IMPROVED TECHNIQUE

On the same 30 birds repeated injections were continued in a different manner. The technique used was simply to make the injection into the vena thoracica externa anywhere along its course on the lateral body wall running posterior and ventrally from the base of the wing (see Figure 13 in text).

1. Pluck feathers from the area.
2. The vein can be enlarged considerably just prior to injection by blocking off the blood flow at a point near the base of the wing.
3. Injection is made in the direction of blood flow.
4. Immediately upon withdrawal of the needle, place forefinger over puncture point and draw the overlying skin snugly in a perpendicular direction to the vein. Hold for about three seconds and release slowly. The clot is so rapid it is often difficult to tell where the puncture was made.

These injections were all continued in the same vein with very little or no evidence of disruption of the blood vessel.

Rationale and Advantages of this Method

(1) Blood in this vein is not under as great pressure as is blood in a large brachial vein and is therefore not as prone to "burst through" a clot at the puncture point.

(2) Even though this vein is smaller than the brachial vein, it wasn't found as hard to hit with a needle because -

(i) overlying skin is not as loose on the body wall as it is on the underside of the wing;

(ii) subcutaneous fascia seems to hold the _vena thoracica externa_ more rigidly than the adventitia holding the brachial vein;

(iii) the vein _thoracica externa_ is also supported firmly underneath by the rib cage and lateral body wall and is not half-submerged in muscle as is the brachial vein.

(3) Wings do not have to be held back to the extent they do for brachial vein injections and as a result the bird is more comfortable and is not as likely to resist the injection.

(4) A perpendicular stretching of the skin across the point of puncture temporarily restricts all blood flow in the vein under the taut skin. This allows the blood to clot at the puncture while the vein is in a 'stretched' condition. Hence, on release, the fibrin mesh of the clot is actually compressed and thereby strengthened. On the other hand, direct pressure over the point of puncture of a vein allows the clot to form while the vessel is in a compressed state, and is actually stretched apart, torn or made considerably weaker upon release. This usually results in a subcutaneous hemorrhage unless considerable time is taken in compression for a strong clot to form.

(5) The bare patch of skin is not exposed to cannibalistic attitudes of other birds because it remains covered by the folded wing.

CONCLUSION

In experiments undertaken by other researchers where, for example, larger needles are required or where there is lack of assistance, I would strongly recommend this technique as an extremely valuable one for repeated injections and one that is definitely superior to conventional brachial vein injections.

APPENDIX 4

Exploratory Observations Based on Visual Inspection of

Spinal Nerve Operated, Sham and Control Birds (3 months old)

Procedure:

Birds were anesthetized with Nembutol. A longitudinal incision was made in the mid-cervical region in order to expose the thymus. Control thymuses were viewed first and then experimental thymuses were compared with the controls visually. Thymuses from operated birds were recorded as being larger (+, ++, +++) or smaller (-, --, ---) in comparison to the control thymuses. Following observations, incisions were sutured with 3-0 surgical silk and the birds were given the usual post-operative care.

Results (Summary)

Thymus Size	Operated	Sham Controls	Total
Normal or larger	5	11	16
Smaller	8	1	9
Total	13	12	25

APPENDIX 5

Results of Methods Used to Confirm Sympathectomy

1. Observable Effect on Errector Pili Muscles of the Head

Immediately after sympathectomy a lack of control of errector pili muscle is noticeable on the head feathers. In Figure 14, head feathers 'stand up' on the side of the head on which sympathectomy was performed. As time progresses, these feathers take on a 'ruffled' appearance on the affected side of the head. In bilaterally ganglionectomized chicks the same effect is noticed over the entire head.

2. Observable Effect on Eyelids Following Sympathectomy: Second Day

The chick exhibits a noticeable effect on eyelid of the operated side. Both upper and lower eyelids 'droop' and lack tonus (on affected side only). Eyelids, however, are still functional on both eyes as are both nictitating membranes. Dilation of the sympathectomized pupil tends to be of a greater degree than that of the pupil of the opposite eye where no sympathectomy took place. However, pupilary reflexes to light (constriction) seem to be normal. These effects of sympathectomy persist throughout the life of the bird.

3. Histology of Sympathetic and Spinal Ganglia

(a) Thionin stain of sympathetic and spinal ganglion confirmed that nerve cell bodies of sympathetic ganglia are evenly dispersed throughout the ganglion whereas spinal ganglia nerve cell bodies are congregated at the periphery of the ganglion.

(b) Azure A and Methylene Blue stains were also used to reveal anatomical morphology of spinal and sympathetic ganglia.

(c) Electron Microscopy. Spinal ganglion and sympathetic ganglion were compared.

4. Effect of Sympathetic Trunk Lesions

Birds with unilateral and bilateral cervical sympathetic trunk lesions exhibited the same 'eyelid droop' effect as did ganglionectomized birds. Photographs taken at nine weeks clearly reveal the same effect persisting on the affected side. Both upper and lower eyelids lack tonus and droop downward. Feathers appear ruffled and tend to 'stick up' in the same manner on the head as they did on chicks which were unilaterally ganglionectomized. The only exception here is that this appearance continued further down the neck.

APPENDIX 6

Fluothane Anesthetic (Inhalation)

Fluothane anesthetic proved to be very successful when used with very young chicks. In addition to it being nonexplosive and nonflammable, it is particularly useful in that its 'safe' concentration range is considerably wider than that of Combutol.

The fluothane in liquid form was injected into a simple oxygen administering device at the rate of .16 ml/min. A slightly higher induction concentration is useful to promote rapid anesthesia.

(Fluothane is distributed by Ayerst Laboratories Ltd.)

APPENDIX 7

Original Raw Data

EXPERIMENT I : Stimulation of the Female Thymus by Thyroxin

Group	Initial Body Weight (gm)		Final Body Weight (gm)		Thymus Weight (gm)	
	Arithmetic	Logarithm	Arithmetic	Logarithm	Arithmetic	Logarithm
Male Controls			1600	3.20412	6.84	0.83506
			1365	3.13513	6.30	0.79934
			1535	3.18611	6.52	0.81425
			1400	3.14613	5.29	0.72346
			1530	3.18469	6.56	0.81690
			1635	3.21352	7.70	0.88649
Female Controls			1130	3.05308	7.59	0.88024
			930	2.96848	4.90	0.69020
			1220	3.08636	5.43	0.73480
			1260	3.10037	4.85	0.68574
			1115	3.04727	3.39	0.53020
			1185	3.07372	3.90	0.59106

EXPERIMENT I (Cont'd)

Group	Initial Body Weight (gm)		Final Body Weight (gm)		Thymus Weight (gm)	
	Arithmetic	Logarithm	Arithmetic	Logarithm	Arithmetic	Logarithm
Sham Injected Males	1050	3.02119	1445	3.15987	4.62	0.66464
	1000	3.00000	1215	3.08458	2.49	0.39620
	1100	3.04139	1360	3.13354	4.30	0.63347
	1150	3.06070	1565	3.19451	6.11	0.78604
	1050	3.02119	1490	3.17319	8.14	0.91062
	1000	3.0000	1460	3.16435	8.57	0.93298
Sham Injected Females	700	2.84510	1085	3.03543	5.54	0.74351
	900	2.95424	1110	3.04532	4.78	0.67943
	870	2.93952	1105	3.04336	2.16	0.33445
	1000	3.0000	1285	3.10890	2.35	0.37107

EXPERIMENT I (Cont'd)

Group	Initial Body Weight (gm)		Final Body Weight (gm)		Thymus Weight (gm)	
	Arithmetic	Logarithm	Arithmetic	Logarithm	Arithmetic	Logarithm
Thyroxin Injected Males	1000	3.000	1110	3.04727	4.12	0.61490
	1050	3.02119	1380	3.13988	5.31	0.72509
	1250	3.09691	1500	3.17609	6.56	0.81690
	1250	3.09691	1380	3.13988	5.60	0.74819
	1050	3.02119	1360	3.13354	4.20	0.62325
	1150	3.06070	1555	3.17754	10.72	1.02019
	1200	3.07918	1475	3.16879	6.54	0.81558
	1300	3.11394	1575	3.19728	5.84	0.76641
	1100	3.04139	1360	3.13354	8.25	0.91645
	1200	3.04139	1510	3.17898	8.10	0.90849
	1050	3.02119	1410	3.14922	10.60	1.02531
	1050	3.02119	1305	3.11561	6.78	0.83123

EXPERIMENT I (Cont'd)

Group	Initial Body Weight (gm)		Final Body Weight (gm)		Thymus Weight (gm)	
	Arithmetic	Logarithm	Arithmetic	Logarithm	Arithmetic	Logarithm
Thyroxin Injected Females	800	2.90309	855	2.93197	5.62	0.74974
	900	2.95424	1160	3.06446	8.75	0.94201
	850	2.92942	1020	3.00860	5.95	0.77452
	850	2.92942	1045	3.01912	10.83	1.03463
	900	2.95424	1180	3.07188	8.31	0.91960
	950	2.97772	955	2.97795	6.67	0.82413
	900	2.95424	1115	3.04727	6.96	0.84261
	900	2.95424	1170	3.06819	8.41	0.92480

EXPERIMENTS II, III & IV : Effect of Spinal Nerve Denervations

Group	Initial Body Weight (gm)		Final Body Weight (gm)		Thymus Weight (gm)	
	Arithmetic	Logarithm	Arithmetic	Logarithm	Arithmetic	Logarithm
Male Controls (Pilot Experiment)	2300	3.36173	2370	3.37475	2.56	0.40824
	1975	3.29557	2000	3.30103	2.47	0.39270
Female Controls (Pilot Experiment)	1760	3.24551	1720	3.23553	0.90	9.95424-10
	1470	3.16732	1360	3.13354	.75	9.87506-10
Sham Operated Uninjected Males (Pilot Experiment)	1765	3.24674	2330	3.36736	1.51	0.17898
	1940	3.28780	1955	3.29115	2.33	0.36736
	1760	3.24551	1740	3.24055	2.5	0.39794
Sham Operated Uninjected Female (Pilot Experiment)	1825	3.26126	1860	3.26951	1.16	0.06446
Bilateral Spinal Nervectomy, Uninjected Males (Pilot Experiment)	2150	3.33244	2050	3.31175	.88	9.94448-10
	2250	3.35218	2115	3.32531	1.10	0.04139

EXPERIMENTS II, III & IV (Cont'd)

Group	Initial Body Weight (gm)		Final Body Weight (gm)		Thymus Weight (gm)	
	Arithmetic	Logarithm	Arithmetic	Logarithm	Arithmetic	Logarithm
Bilateral Spinal Nervectomy, Uninjected Females (Pilot Experiment)	1630	3.21219	1635	3.21352	.99	9.99564-10
	1295	3.11227	1280	3.10721	.72	9.85733-10
	1580	3.19866	1510	3.17898	1.43	0.15534
Unoperated, Thyroxin Injected Males (Pilot Experiment)	2080	3.31806	2080	3.31806	4.80	0.68124
	1800	3.25527	1785	3.25164	1.30	0.11394
Unoperated, Thyroxin Injected Females (Pilot Experiment)	1900	3.27875	1685	3.22660	2.32	0.36549
	1350	3.13033	1275	3.10551	1.90	0.27875
Sham Operated Thyroxin Injected Males (Pilot Experiment)	1940	3.28780	1830	3.26245	2.10	0.32222
	1800	3.25527	1645	3.21617	2.51	0.39967
	2110	3.32428	2000	3.30103	2.79	0.44560
Sham Operated Thyroxin Injected Females (Pilot Experiment)	1680	3.22531	1560	3.19312	3.18	0.50243

EXPERIMENTS II, III & IV (Cont'd)

Group	Initial Body Weight (gm)		Final Body Weight (gm)		Thymus Weight (gm)	
	Arithmetic	Logarithm	Arithmetic	Logarithm	Arithmetic	Logarithm
Bilateral Spinal Nervectomy, Thyroxin Injected Males (Pilot Experiment)	2110	3.32428	2130	3.36173	5.23	0.71850
	1860	3.26951	1510	3.17898	1.25	0.09691
	1860	3.26951	1575	3.19728	1.95	0.29003
	2010	3.32222	1675	3.22401	1.84	0.26482
Bilateral Spinal Nervectomy, Thyroxin Injected Females (Pilot Experiment)	1480	3.17026	1355	3.13194	2.22	0.34635
	1445	3.15987	1270	3.10380	2.43	0.38561
	1640	3.21484	1400	3.14613	5.49	0.73957
No Operation, No Thyroxin, Males	1160	3.06446	1550	3.19033	7.36	0.86688
	1605	3.20548	2020	3.30535	7.12	0.85248
	1380	3.13988	1620	3.20952	6.41	0.80686
	800	2.90309	1060	3.02531	3.84	0.58433
No Operation, No Thyroxin, Females	1125	3.04115	1380	3.13988	1.00	0.00000
	1240	3.09342	1460	3.16435	3.32	0.50786
	1000	3.0000	1195	3.07737	5.04	0.70243
	1135	3.05500	1270	3.10380	1.26	0.10037

EXPERIMENTS II, III & IV (Cont'd)

Group	Initial Body Weight (gm)		Final Body Weight (gm)		Thymus Weight (gm)	
	Arithmetic	Logarithm	Arithmetic	Logarithm	Arithmetic	Logarithm
No Operation, Thyroxin Injected, Males	955	2.98000	1480	3.17026	14.66	1.16613
	1390	3.14301	1635	3.21352	6.18	0.79099
	1475	3.16879	1690	3.22789	5.04	0.70243
	1460	3.16435	1750	3.24304	8.44	0.92634
	1480	3.17026	1810	3.25768	6.20	0.79239
	1200	3.07918	1550	3.19033	5.06	0.70415
No Operation, Thyroxin Injected, Females	690	2.83885	1100	3.04532	8.93	0.95085
	1150	3.06070	1360	3.13354	4.61	0.66370
	980	2.99123	1180	3.07188	5.36	0.72916
	1060	3.02531	1310	3.11727	3.22	0.50786
	1205	3.07099	1480	3.17026	7.66	0.88423
Unilateral Spinal Nervectomy, No Thyroxin, Males	1710	3.23300	1990	3.29885	4.53	0.65610
	1340	3.12710	1720	3.23553	3.94	0.59550
	1660	3.22011	1900	3.27875	5.59	0.74741
	1370	3.13672	1650	3.21748	4.22	0.62531

EXPERIMENTS II, III & IV (Cont'd)

Group	Initial Body Weight (gm)		Final Body Weight (gm)		Thymus Weight (gm	
	Arithmetic	Logarithm	Arithmetic	Logarithm	Arithmetic	Logarithm
Unilateral Spinal Nervectomy, No Thyroxin, Females	990	2.99564	1155	3.06258	2.72	0.43457
	850	2.92942	1080	3.03342	6.05	0.78176
	1175	3.06004	1470	3.16732	2.40	0.38021
Unilateral Spinal Nervectomy, Thyroxin Injected, Males	1730	3.23805	2000	3.30103	11.29	1.04269
	1540	3.18752	1650	3.21748	8.17	0.91222
	1750	3.24304	2025	3.30643	9.46	0.97589
	1550	3.19033	1730	3.23805	5.62	0.74974
Unilateral Spinal Nervectomy, Thyroxin Injected, Females	880	2.94448	1255	3.09864	10.68	1.02857
	1320	3.12057	1510	3.18909	2.26	0.35411
	1080	3.03342	1315	3.11893	2.07	0.31597
Bilateral Spinal Nervectomy, No Thyroxin, Males	1100	3.04139	1460	3.16435	7.20	0.85733
	1425	3.15381	1740	3.24055	6.42	0.80754
Bilateral Spinal Nervectomy, No Thyroxin, Females	1225	3.09691	1490	3.17319	1.85	0.26717
	1090	3.03743	1320	3.12057	2.03	0.30750

EXPERIMENTS II, III & IV (Cont'd)

Group	Initial Body Weight (gm)		Final Body Weight (gm)		Thymus Weight (gm)	
	Arithmetic	Logarithm	Arithmetic	Logarithm	Arithmetic	Logarithm
Bilateral Spinal Nervectomy, Thyroxin Injected, Males	960	2.98227	1410	3.14922	6.82	0.83378
	1540	3.17590	1770	3.24797	10.00	1.0000
Bilateral Spinal Nervectomy, Thyroxin Injected, Females	1080	3.03342	1310	3.11727	2.59	0.41330
	1140	3.05690	1330	3.12385	6.40	0.80618
	1060	3.02531	1300	3.11394	4.09	0.61172
	1125	3.04115	1185	3.07372	1.29	0.11059

EXPERIMENT V : Effect of Superior Cervical Sympathectomy

Group	Initial Body Weight (gm)		Final Body Weight (gm)		Thymus Weight (gm)	
	Arithmetic	Logarithm	Arithmetic	Logarithm	Arithmetic	Logarithm
Unoperated, No Thyroxin, Males	1480	3.17026	1510	3.17898	5.83	0.76567
	1360	3.13354	1725	3.23679	3.55	0.55023
	1370	3.13672	1685	3.22660	10.05	1.00217
	1430	3.15534	1695	3.22917	5.28	0.72263
	1280	3.10721	1580	3.19866	4.71	0.67302
	1290	3.11059	1585	3.19003	6.68	0.82473
	1165	3.06633	1525	3.18327	6.03	0.78032
	1160	3.06446	1580	3.19866	6.07	0.78319
Unoperated, No Thyroxin, Females	1230	3.08991	1430	3.15534	4.25	0.62839
	1080	3.03342	1450	3.16137	3.50	0.54407
	1000	3.00000	1275	3.10551	6.74	0.82866
	930	2.96949	1175	3.06004	5.56	0.74507
	865	2.93702	1180	3.07188	7.58	0.87967
	1065	3.21748	1290	3.11059	6.08	0.78390
	945	2.97543	1110	3.04532	7.42	0.87040
	925	2.96614	1220	3.08636	5.83	0.76567
	850	2.92942	1070	3.02938	6.00	0.77815
	940	2.97313	1600	3.70412	6.66	0.81954

EXPERIMENT V (Cont'd)

Group	Initial Body Weight (gm)		Final Body Weight (gm)		Thymus Weight (gm)	
	Arithmetic	Logarithm	Arithmetic	Logarithm	Arithmetic	Logarithm
Unoperated, Thyroxin Injected, Males	1190	3.04555	1485	3.17173	6.40	0.80618
	980	2.99123	1065	3.02735	1.70	0.23045
	1140	3.05690	1535	3.18611	6.70	0.82607
	1500	3.17609	1730	3.23805	7.32	0.86451
	1400	3.14613	1780	3.25042	7.65	0.88366
	1420	3.15229	1625	3.20085	7.68	0.88536
	1220	3.08636	1330	3.12385	2.95	0.46982
	1530	3.18469	1650	3.21748	5.79	0.76268
Unoperated, Thyroxin Injected, Females	895	2.95182	1100	3.04139	6.19	0.79169
	810	2.90849	1040	3.01703	9.72	0.98767
	690	2.83885	920	2.96379	4.23	0.62634
	890	2.94939	1070	3.02938	8.90	0.94939
	890	2.94939	1110	3.04532	9.40	0.97313
	855	2.93197	1050	3.02119	7.37	0.86747
	1270	3.09691	1320	3.12057	1.92	0.28330
	1040	3.01703	1270	3.10280	7.38	0.86806
	630	2.79934	1000	3.0000	5.11	0.70842
	1365	3.13513	1410	3.14922	1.93	0.28556

EXPERIMENT V (Cont'd)

Group	Initial Body Weight (gm)		Final Body Weight (gm)		Thymus Weight (gm)	
	Arithmetic	Logarithm	Arithmetic	Logarithm	Arithmetic	Logarithm
Unilateral Sympathectomy, No Thyroxin, Males	1540	3.18752	1830	3.26245	4.76	0.67761
	1340	3.12710	1650	3.21748	6.58	0.81823
	1380	3.13988	1550	3.19033	5.18	0.71433
	1310	3.11727	1525	3.18327	8.42	0.92531
	1360	3.13354	1650	3.21748	4.40	0.64345
	1530	3.18469	1670	3.22272	3.02	0.48001
	1350	3.13033	1700	3.23045	8.04	0.90526
	1975	3.29557	2160	3.33445	5.57	0.74586
	1230	3.08991	1500	3.17609	5.15	0.71181
	1090	3.03743	1360	3.13354	6.41	0.80686
Unilateral Sympathectomy, No Thyroxin, Females	1090	3.03743	1375	3.13830	2.65	0.42325
	980	2.99123	1080	3.03543	5.72	0.75740
	930	2.96848	1210	3.08458	6.08	0.78390
	905	2.95665	1235	3.08167	5.71	0.75664
	800	2.90309	1075	3.02141	5.56	0.74507

EXPERIMENT V. (Cont'd)

Group	Initial Body Weight (gm)		Final Body Weight (gm)		Thymus Weight (gm)	
	Arithmetic	Logarithm	Arithmetic	Logarithm	Arithmetic	Logarithm
Unilateral Ganglionectomy, Thyroxin Injected, Males	1520	3.18184	1880	3.27416	7.98	0.90200
	1290	3.11059	1635	3.21352	6.90	0.83885
	1090	3.03743	1425	3.15381	7.51	0.87564
	1220	3.08636	1250	3.09691	5.11	0.70842
	1490	3.17319	1690	3.22789	6.24	0.79518
	1170	3.06819	1500	3.17609	10.46	1.01912
	1370	3.13672	1650	3.21748	4.81	0.68215
	1170	3.06819	1310	3.11727	7.84	0.89432
	890	2.94939	1205	3.07099	5.92	0.77232
	1050	3.02119	1390	3.14301	5.70	0.75587
Unilateral Ganglionectomy, Thyroxin Injected, Females	840	2.92428	1040	3.01703	7.65	0.88366
	820	2.91381	1150	3.06070	8.20	0.91381
	875	2.94201	1040	3.01703	10.31	1.01284
	960	2.98227	1160	3.06446	7.53	0.87679
	950	2.97772	1095	3.03941	8.35	0.92169

EXPERIMENT "V" (Cont'd)

Group	Initial Body Weight (gm)		Final Body Weight (gm)		Thymus Weight (gm)	
	Arithmetic	Logarithm	Arithmetic	Logarithm	Arithmetic	Logarithm
Bilateral Sympathectomy, No Thyroxin, Males	1360	3.13354	1500	3.17609	2.42	0.38382
	1220	3.08636	1425	3.15381	3.75	0.57403
	1415	3.14076	1690	3.22789	5.54	0.74351
	1220	3.08636	1530	3.18469	8.43	0.92583
	970	2.98677	1385	3.13145	5.92	0.77232
	1250	3.09691	1670	3.22272	8.10	0.90840
	1235	3.08167	1490	3.17319	3.53	0.54777
	1180	3.07372	1595	3.20276	6.78	0.83123
	1240	3.09342	1560	3.19312	5.62	0.74974
Bilateral Sympathectomy, No Thyroxin, Females	980	2.99123	1075	3.02141	2.47	0.39270
	1160	3.06446	1555	3.19173	5.23	0.71850
	885	2.94694	1050	3.02119	4.87	0.68753
	770	2.88649	1070	3.02938	5.56	0.74507
	930	2.96848	1090	3.03743	4.75	0.67669
	660	2.81954	710	2.85126	0.94	9.97313-10

EXPERIMENT V (Cont'd)

Group	Initial Body Weight (gm)		Final Body Weight (gm)		Thymus Weight (gm)	
	Arithmetic	Logarithm	Arithmetic	Logarithm	Arithmetic	Logarithm
Bilateral Sympathectomy, Thyroxin Injected, Males	1045	3.01912	1480	3.17026	6.28	0.79796
	1180	3.07188	1650	3.21748	7.18	0.85612
	1150	3.06070	1640	3.21484	14.48	1.15077
	1275	3.10551	1575	3.19728	12.12	1.08350
	1580	3.19866	1770	3.24797	8.58	0.92349
	1250	3.09691	1300	3.11394	5.06	0.70415
	1275	3.10551	1450	3.16137	8.01	0.90363
	1260	3.10209	1455	3.16286	6.32	0.80072
	1230	3.08167	1430	3.15534	5.87	0.76864
Bilateral Sympathectomy, Thyroxin Injected, Females	955	2.98000	1195	3.07737	10.39	1.01662
	980	2.99123	1230	3.08991	9.71	0.98722
	955	2.98000	1200	3.07918	10.90	1.03743
	820	2.91381	1130	3.05308	9.06	0.95713
	870	2.98677	1110	3.04532	4.97	0.69636
	940	2.97313	1120	3.04922	8.31	0.91960